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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our goal is to understand mechanisms by which neurotoxicity destroys cells in the substantia nigra. Our hypothesis is that c-JUN kinases (JNK), which can contribute to neuronal death, mediate neurodegeneration in the substantia nigra after exposure to MPTP or excitotoxicity. Results in year 3 indicated that mice lacking JNK 1 or JNK 3 lack neuroprotection against MPTP neurotoxicity in the substantia nigra and excitotoxicity in striatal neuronal cultures. Results support a role for JNK proximal to mitochondrial initiation of apoptosis and overlapping function of JNK isoforms. Furthermore, in another model of neuronal apoptosis, p38 and not JNK serves as a target for MAP kinase dependent apoptosis. Additional studies indicate that neuronal apoptotic mechanisms can lead to increased aggregation of proteins in selected neurodegenerative diseases. Preliminary studies on stem cell transplantation were extended to show that knockout of an immune related gene improves survival and integration of stem cells in substantia nigra. Our studies offer a revised signaling system for neuronal apoptosis and new approach to improve survival of bone-marrow derived stem cells in damaged brain.

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Table of Contents

INTRODUCTION

Subject and purpose. Cell death in the substantia nigra causes Parkinson's disease. Most causes of Parkinson's disease are unknown, but some patients have a hereditary form (mutations in α-synuclein or parkin) or a neurotoxin dependent cause, such as MPTP (1,2). Recent research has described aberrant protein aggregation as an underlying pathogenesis in Parkinson's disease (3). This laboratory is involved in three areas of research in neurotoxicity models of Parkinson's disease: mechanisms of MPTP-dependent apoptotic cell death, mechanisms of aberrant protein aggregation leading to neurodegeneration and new strategies to treat neurotoxic cell death with bone marrow derived stem cells. These three research directions share apoptosis, also called programmed cell death. Apoptosis is strongly implicated in neurodegeneration in Parkinson's disease and other neurodegenerative diseases, such as Huntington's disease (4-7). In apoptosis, neurons recruit intracellular signals that ultimately lead to DNA cleavage, nuclear shrinkage and rapid engulfment of dying cells by glia. The rapid removal of cells protects the brain from non-specific spillage of cell contents. Furthermore, neuronal survival might be improved by factors that avert apoptotic stimuli.

Scope. How neurons initiate and signal apoptotic cues remains a rapidly evolving field in science. Contributing to apoptosis are extracellular cues, such as the binding of FAS ligand to some cells, and intracellular signals, including the release of cytochrome C from mitochondria. Regulation of release of cytochrome C has become increasingly important in the past two years, with the recognition that cytochrome C in the cytoplasm constitutes a pivotal signal to activate rapid apoptotic events. Our original hypothesis was that cytochrome C activates apoptosis through the enzyme caspase 3. Another important enzyme, called c-JUN kinase (JNK), mediates several additional intracellular pathways in apoptosis (8-10). JNK could affect caspase 3 activity (a point of integration of pathways between cytochrome C and extracellular events) and also initiates apoptosis through its phosphorylation of c-JUN. We hypothesized that mitochondrial impairment released cytochrome C and activated JNK, thereby promoting apoptosis.

The rationale underlying hypothesis testing is that results can be placed in a proper framework whether experiments support or refute the original hypothesis. Our findings indicated that mitochondrial impairment does not affect JNK mediation of neuronal death. Indeed, our results favor a role for JNK (or an isoform) in modifying mitochondrial responses to neurotoxins. This idea will be developed in the Results section.

An emerging concept in Parkinson's disease is that protein aggregation lies at the center of pathogenesis of many neurodegenerative diseases (3). Abnormal proteins, or products of proteolysis, forms proto-fibrillar structures, which ultimately damage neurons. Mechanisms probably vary. In Alzheimer's disease, protein aggregation generally is extraneuronal, whereas in Parkinson's disease (Loewy bodies) and Huntington's disease the aggregation is intracellular. We have studied protein aggregation of mutant huntingtin and found that cleaved portions of the mutant huntingtin are more likely to form aggregates than is the intact protein (11). Thus, how cells handle

abnormal proteins might be a new focus of study for therapy in neurodegenerative diseases.

We also started a new series of projects on cell replacement in neurodegenerative diseases, especially Parkinson's disease. The impetus for this new direction originated in the DOD Workshop, Potomac, MD, March, 2001. Presentations revealed that neuronal transplantation could occur through stem cells or fetal transplantation, but that survival of the transplanted cells was insufficient to be effective. The assumption in the presentations was that the brain is an immune-privileged site, so that other factors (neuronal integration, vascular considerations) were at the core of the inadequate survival. However, we had reported that in Huntington's disease microglia surrounded areas of neuronal loss (12). Microglia have immune competence. Therefore, we postulated that targeted changes in host immune competence might improve stem cell survival. We will show in the Results pilot data on reversal of motor impairment by stem cell implantation. Our research team comprises Dale Greiner (a transplantation immunologist in the Department of Medicine at U Mass), Dr. Leonard Schulz (an expert in mouse genetics and mouse modeling of disease at Jackson Labs, Bar Harbor, Maine) and myself.

Background summary. Our overall goal is to prevent or repair neurotoxin induced damage of the substantia nigra. It is our intention that information garnered from this research can be applied to treatment for Parkinson's disease. We originally hypothesized that the enzyme JNK is central to apoptosis induced by the neurotoxin MPTP (13-16). Our current data modifies this hypothesis, placing JNK proximal to MPTP damage of mitochondria and subsequent apoptosis. We extended this research to include studies on another neurotoxin, 3-nitroproprionic acid. Results from this proposal and companion experiments in our laboratory point to changes in protein handling leading to aggregation as a critical step causing neurodegeneration. We therefore pursued proteolysis to understand mechanisms of neurodegenerative disease. We also initiated studies to improve survival of replacement of neurotoxin-damaged neurons, by altering host immune systems. Altogether, over the past 3.5 years, we expanded our program to arrive at a new signal transduction mediating neuronal apoptosis, a role for proteolysis in neurodegeneration, and determination of changes in host immune defense to improve stem cell survival. The DOD supported each of these directions.

BODY

Experiments were performed to study the effects of JNK absence on neuronal survival in the substantia nigra after MPTP administration. Experiment designs were modified based on information imparted at the DOD conference. Comparative studies on another mitochondrial toxin, 3-NP, were undertaken in striatal neurons with or without JNK1 or JNK3. Additional studies on caspase 3 (a key enzyme in apoptosis) and NMDA receptor activation in HD were completed. Studies on protein aggregation in neuronal survival were continued. Studies on stem survival in brain were continued and additional preliminary data provided.

Experimental methods. *Animals*. We used JNK1, JNK2 and JNK3 knockout mice for the MPTP study. Control mice were siblings with both alleles for each JNK isoform, as determined by PCR. Mice were 8-12 weeks old in these studies, a change from prior experiments (where mice were older than 6 months). Dr. Serge Przedborski recommended the change at the DOD conference and in private communication, in order to improve animal survival after MPTP and obtain more consistent neurotoxic effects. Animals were injected with MPTP or vehicle in a glove box in a P3 facility and were kept alive for 7 days before immunohistochemical analysis. For primary neuronal cultures, JNK1 and JNK 3 knockout mice were super-ovulated and day 15 fetal gestation fetuses were harvested to make primary striatal neuronal cultures, to be tested for 3-NP toxicity. Transgenic Huntington's disease murine models were generated in SJL/C57bl/6 mice (see manuscript). Irradiated rag(-/-) mice were used for stem cell survival studies.

MPTP use. MPTP was injected intraperitoneally. From experiments in year 1, we standardized the dose of MPTP to 20 mg/kg ip x 5days. Animals were allowed to survive 2 additional days.

Immunohistochemistry. Animals were anesthetized with Avertin (0.23 ml/10g bw ip), perfused with 4% paraformaldehyde and post-fixed for 2 hours. Brain sections (50 μm) were cut on a Vibratome, blocked with horse serum and treated with anti-tyrosine monoclonal antibody (Sigma) at a dilution of 1:100. Vectorstain anti-mouse ABC kit was used to detect the diaminobenzidine product. The full substantia nigra was sectioned, with every fourth section used for counting.

Cell analysis. As reported here, cell counting was performed on every fourth section. The observer was blinded to the animal. Stereological counting is used. Dr. DiFiglia will examine the morphology of the neurons (soma size and shape, dendritic size and shape, vacuoles, nuclear shrinkage and overal neuropil labeling) and the intensity of tyrosine hydroxylase immunoreactivity (Sigma scan densitometry).

Primary neuronal cultures. Dr. Genevieve Laforet in the laboratory developed a method of culturing primary striatal neurons. She dissects the primordial striatal buds, lightly triterates the tissue and plates the neurons. After 10 days, the cultures are studied for 3-NP neurotoxicity. 3-NP is added to the culture and the neurons are examined for cell survival at baseline, 24, 48 and 72 hours. Images of surviving neurons are captured on a digital microscope. In separate tests, MTT (mitotracker) is used to measure the integrity of the mitochondria. Four impartial observers count surviving cells.

Proteolysis studies. These studies were performed in collaboration with Dr. Marian DiFiglia at Massachusetts General Hospital. A clonal striatal cell line, X-57, (gift of Dr. A. Heller, U of Chicago) was used. Flag-tagged mutant and wild type huntingtin cDNAs were transfected into the clonal cells. At various time points post transfection, protein from cell extracts was immunoprecipitated with anti-Flag or anti-htt antisera and identified on Western blot. Similar protein analysis was carried out in post-mortem patient striatum. Caspase 3 or calpain treatment, or specific blockade of these enzymes, was performed in vitro and the protein products analyzed on Western blot.

Statistical analysis. Dr. Robert Lew provided the statistical analysis for the transgenic HD murine model. Several parametric and non-parametric tests were used, including Hazard scores, chi-square and ANOVA.

Results.

Positive findings. **Studies on JNK neuroprotection against MPTP administration**. Since the fall of 2001, we doubled the number of mice used in each group: JNK 1 knockout and replete; JNK 3 knockout and replete. Our results indicate a lack of neuroprotection against MPTP in tyrosine hydroxylase neurons in the substantia nigra (Fig. 1). Please note that the original hypothesis was that JNK 3 would offer protection against mitochondrial dependent apoptosis. The JNK 3 protection is based on the finding that JNK 3 knockout mice lack kainate (seizure) induced hippocampal cell death. Our finding indicates that JNK 1 and JNK 3 do not mediate apoptosis after mitochondrial impairment. This is not to say that JNKs have no role in mitochondrially dependent cell death; rather, elimination of JNK 1 and JNK 3 is insufficient to abrogate apoptosis under the conditions of the experiment.

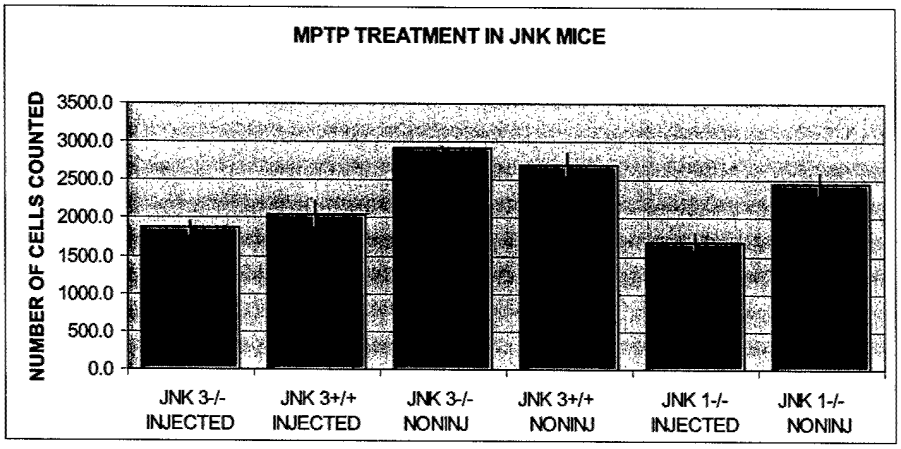

Figure 1. Neuronal loss in the substantia nigra after MPTP administration in JNK replete and JNK knockout mice. JNK replete = +/+. JNK knockout = -/-. N=number of mice. Bars=SE.

We next studied a possible role for JNK in another model of mitochondrial impairment -- with use of 3 nitroproprionic acid (3NP). This compound is a mitochondria toxin. We examined the effect of 3NP in primary striatal neurons in culture. 3NP treatment of primary striatal neurons causes substantial neuronal death after 72 hours. We tested primary striatal neurons from JNK 1 and JNK 3 knockout and

replete mice. Again, no neural protection was found in neurons from knockout mice (Fig. 2). Cell survival was measured by counting live cells in a cell viability assay and by measuring MTT (the more MTT, the more cells survived). Both measurements were consistent.

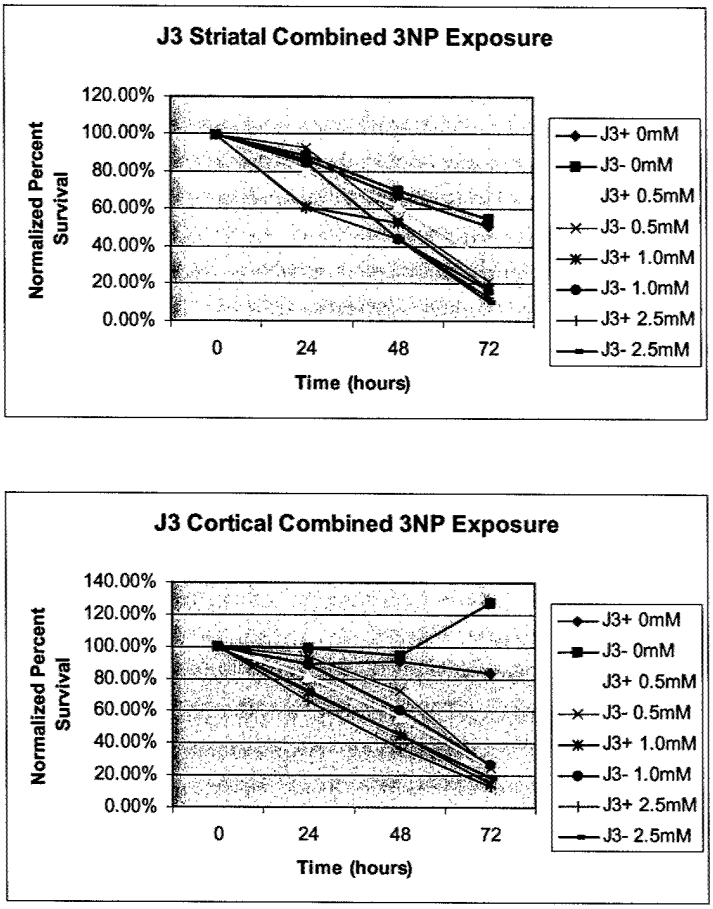

Figure 2. Neuronal survival in JNK replete and JNK knockout primary neurons: effect of 3NP. We studied the effect of the mitochondrial toxin 3NP in primary neuronal cultures from JNK replete and JNK knockout mice (JNK 1 and JNK3). Primary neuronal cultures from gestation age 15 were plated and treated with 3NP on day 4. Cell counts and MTT measurements were made in separate wells at baseline treatment, 24, 48 and 72 hours. We normalized the cell counts and MTT to untreated primary neuronal cultures at the same time points.

In unpublished studies, Dr. Davis's laboratory found that JNK3 is -- by far -- the dominant JNK in the hippocampus. JNK knockout mice have very little JNK remaining in hippocampus. In contrast, substantial amounts of JNK 1 and JNK 2 remain in the substantia nigra and striatum in JNK 3 knockout mice. A possible explanation for the hippocampal protection is that another JNK might be involved in mediating apoptosis in

substantia nigra and striatum. To explore this possibility, we studied mice that lack the gene for JIP 1 (JNK interacting protein). JIP 1 appears to be a scaffolding protein for all three JNK products (17). JIP 1 knockout mice can also exhibit hippocampal neuronal sparing after excitotoxic injury. Thus, JIP 1 knockout is an alternative model to examine roles of JNKs in mediating apoptosis after mitochondrial impairment, such as MPTP in the substantia nigra and 3NP in the striatum. Our data reveal no consistent protection of the JIP1 knockout striatal cells after 3NP treatment.

Stem cell survival in brain: administration of bone-marrow derived stem cells into chemically-lesioned substantia nigra. We continue to study the use of bone-marrow derived stem cells as replacement therapy in immune-privileged hosts. Our main idea is that inadequate survival of neuronal stem cells in brain might be due in part to immune rejection of the cells. Although the brain is considered an immune privileged site, the brain maintains immune competent cells and immune rejection mechanisms. We therefore used genetically less competent mice (rag -/-) as hosts and GFP stem cells (prepared by immunoisolation) as donors. Dr. Leonard Schulz at Jackson Laboratory created the mice, Dr. Dale Greiner at U Mass Med Sch prepared the bone-marrow derived stem cells, and my laboratory prepared chemically lesioned mice (6OHDA in substantia nigra and quinolinic acid in the striatum). Mice were tested for motor response to apomorphine for adequacy of lesions. Stem cells were injected into the striatum or the substantia nigra. Mice were allowed to survive 8 weeks; location of GFP neurons was evaluated. Of the six mice that were lesioned, all exhibited abnormal turning after apomorphine injection, consistent with a severe chemical lesion of either quinolinic acid in the striatum or 6OHDA in the substantia nigra. Of mice receiving stem cells, five had resolution of their abnormal turning. Mice that received striatum injections after quinolinic acid destruction of the striatum showed GFP positive cells in the internal capsule adjacent to the substantia nigra and neuron-like structures with GFP in the substantia nigra (Fig. 3). These studies will be pursued.

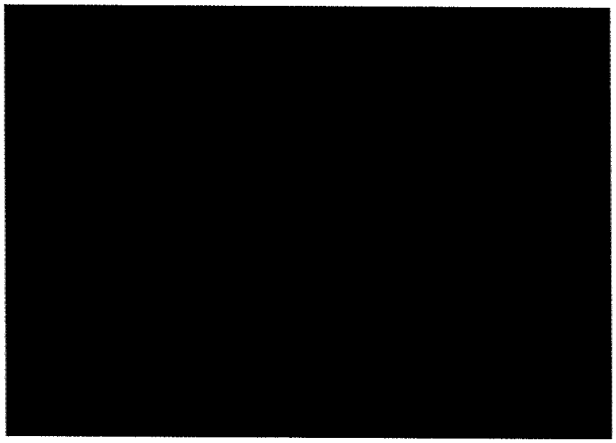

Figure 3. Bone-marrow derived stem cells transplanted into chemically-lesioned striatum: survival of neuron-like cells in the substantia nigra. Bone-marrow derived stem cells from GFP positive donor mice were implanted into the striatum of a chemically-

lesioned mouse (quinolinic acid). After 8 weeks, the brain was evaluated for the survival of GFP positive donor cells. The fluorescent cells in the substantia nigra have the appearance of tyrosine hydroxylase neurons.

Negative findings. We believe that we will ultimately disprove the hypothesis that JNK 3 is a general brain JNK that mediates many forms of apoptosis after neurotoxicity. We are currently revising our ideas, with consideration that the JNK system is redundant in many, but not all, brain regions. We are currently comparing mitochondrially induced neuronal death with excitotoxic agents.

Problems in accomplishing tasks. We adjusted our protocol to use younger mice with different MPTP injection protocols. We now have had improved survival. Also, we originally had few stem cells enter the brain after their administration in the general circulation. We changed our protocol to introduce the stem cells in brain regions of chemically lesioned mice. Our early results indicate improvement in behavior of the mice and possible integration of GFP cells in the fabric of the substantia nigra.

KEY RESEARCH ACCOMPLISHMENTS

- Placement of c-JNK involvement in apoptosis is proximal to mitochondria.

- p38, rather than JNK, is a mediator of MAO-A dependent apoptosis in PC12 cells.

- Caspase 3 cleaves mutant huntingtin, which might increase huntingtin aggregation. Calpain might also have a role in huntingtin proteolysis. Aggregation of proteins that cause neurodegenerative disease (e.g., as in HD and PD) might be processed by caspases and calpains.

- Mutant huntingtin increases striatal neuron response to NMDA activation, with increases in Ca^{++} flux.

- Direct administration of bone-marrow derived stem cells into chemically lesioned brain can reverse behavioral abnormalities and lead to donor cell neural integration, in immune altered rag (-/-) mice. Specific immune responses in brain might be targets for treatment to improve stem cell neural therapy.

REPORTABLE OUTCOMES (2001 and 2002)

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CONCLUSIONS

Our original hypothesis is that JNK mediates degeneration of substantia nigra neurons after treatment with MPTP. Our results from current investigations indicate that JNK participates in apoptosis proximal to mitochondrial impairment, rather than after mitochondrial involvement. We did not find neuroprotection against MPTP or other mitochondrial toxins in animals and primary neuronal cultures lacking either JNK 1 or JNK 3. In our view, use of JNK antagonists would not be expected to protect against MPTP neurotoxicity. In studies supported by this grant, another MAP kinase target, p38, does have a prominent role in MAO-A mediated neurotoxicity in PC12 cells. JNK does not transduce this form of apoptosis. Our companion studies on mutant huntingtin indicate that mediators of apoptosis, such as caspase 3, might increase aberrant protein aggregation. Protein aggregation, especially protofibrillation, is implicated in Parkinson's disease. Finally, stem cell transplantation has been considered a treatment option for neurodegenerative diseases. Our initial findings suggest that attenuation of brain immunity mechanisms, such as evident in our knockout mice, might improve stem cell survival and integration into the substantia nigra.

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